

## Low CD4<sup>+</sup> T-Lymphocyte Values in Human Immunodeficiency Virus-Negative Adults in Botswana

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**CD4<sup>+</sup>-lymphocyte counts (LCs) play a crucial role in the management and monitoring of HIV infection. Variability in CD4<sup>+</sup> LCs has been reported to occur as a result of measurement techniques and/or biological variations. We report on the CD4<sup>+</sup> LCs of healthy human immunodeficiency virus (HIV)-seronegative adults in Botswana. Samples were obtained from HIV-seronegative blood donors. The median CD4<sup>+</sup> LC was 726 cells/mm<sup>3</sup> (for females, 782 cells/mm<sup>3</sup>; for males, 698 cells/mm<sup>3</sup>). The median CD8<sup>+</sup> LC was 488 cells/mm<sup>3</sup> (for females, 494 cells/mm<sup>3</sup>; for males, 485 cells/mm<sup>3</sup>). The median CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio was 1.57 (for females, 1.66; for males, 1.51). Our findings of low CD4<sup>+</sup> LCs among HIV-negative adults in Botswana are significant and have important implications for the management of HIV disease in the population of this sub-Saharan African country.**

CD4<sup>+</sup>-lymphocyte counts (LCs) are recognized as the most important measurement of overall human immunodeficiency virus (HIV)-induced immune impairment (27). CD4<sup>+</sup> LCs are an established predictor of disease-free survival and serve as an important guide in the decision to begin prophylactic interventions (26). In addition, CD4<sup>+</sup> LCs help to determine when to start combination antiretroviral therapy in routine clinical practice (8, 13). CD4<sup>+</sup> LCs also serve to monitor immune recovery in patients receiving antiretroviral therapy (2).

Variability in CD4<sup>+</sup> LCs among healthy persons has been widely reported and has been attributed both to biological influences and to differences in the methodologies used for T-cell enumeration. Biological factors that influence CD4<sup>+</sup> LCs include gender (22, 32), age (15, 38), exercise and diurnal variation (19, 23, 30), pregnancy (37, 41), and comorbid medical conditions. Variations in the distributions of white blood cell counts and, specifically, CD4<sup>+</sup>-cell counts among ethnic groups have also been reported. Published reference ranges for CD4<sup>+</sup> LCs in HIV-negative populations from Africa and Asia vary widely (1, 10, 20, 28, 29, 39, 40, 43, 44, 45). Importantly, some of the reported values are significantly lower than the values established for North American and European cohorts, the population in which the kinetics of CD4<sup>+</sup> LC decline in HIV disease are best documented (14, 29, 39).

Flow cytometry, the present reference method used to count the absolute numbers of CD4<sup>+</sup> T cells, is a rapidly evolving diagnostic approach with various permutations in the techniques used. These variations highlight the need for a standardized methodology to ensure that precise and reproducible CD4<sup>+</sup> LCs are obtained. Significant interlaboratory CD4<sup>+</sup> LC

variability has been reported by use of the conventional two-step procedure, which couples the percentage of CD4<sup>+</sup> cells obtained by flow cytometry with the absolute lymphocyte counts obtained with a hematology analyzer (dual-platform technology) (3, 7, 11). Recent recommendations from the Division of AIDS, U.S. National Institutes of Health, favor the use of single-platform methods which directly count the absolute CD4<sup>+</sup> LCs from a single tube (4, 6, 12, 34, 35).

Botswana, where the prevalence of HIV infection is one of the highest in the world, began a national antiretroviral treatment program in the public sector in January 2002. The Botswana Ministry of Health initially chose to offer highly active antiretroviral therapy (HAART) to all symptomatic HIV-infected citizens (those with an AIDS-defining illness) and/or those with a CD4<sup>+</sup>-T-lymphocyte count of less than 200 cells/mm<sup>3</sup> (5). International consensus guidelines for adults recommend that physicians consider initiating HAART when CD4<sup>+</sup> LCs are higher (less than 350 cells/mm<sup>3</sup>), especially in patients with plasma HIV-1 RNA levels greater than 50,000 to 100,000 copies/ml (9) or with a rapid decline in CD4<sup>+</sup> LCs. Botswana's decision to initiate HAART when CD4<sup>+</sup> LCs are lower was influenced largely by logistical and financial considerations, with HAART initially offered to those who clinically needed therapy the most. The normal ranges of CD4<sup>+</sup> LCs in HIV-negative individuals have not yet been described in this region of Africa. Differences in normal cell count ranges from this region of the world may lead to a local reevaluation of treatment guidelines that have been based on the findings of studies with non-African cohorts.

The goal of this study is to describe CD4<sup>+</sup> LC reference ranges among HIV-negative adults in Botswana by evaluating blood specimens obtained from two distinct populations: (i) blood donors and (ii) participants of the Botswana 2001 HIV Sentinel (Seroprevalence) Surveillance. Sentinel surveillance

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TABLE 1. CD4<sup>+</sup> lymphocyte counts for HIV-negative adults in Botswana

Group and sex	No. of subjects	CD4 <sup>+</sup> LC (no. of cells/mm <sup>3</sup> )					
		Median	Range	2.5th–97.5th percentile	Mean	SD	95% CI for mean
Blood donor							
Female	143	786	344–1,558	438–1,328	827	245	787–868
Male	294	698	171–1,652	366–1,252	725	238	698–753
Both	437	726	171–1,652	366–1,318	759	245	736–782
Sentinel surveillance							
Female	207	612	152–1,245	276–1,062	626	200	599–653
Male	44	591	208–1,282	233–1,276	626	246	551–701
Both	251	599	152–1,282	275–1,114	626	208	600–652

has been carried out annually in Botswana since 1992 to monitor the course of the HIV epidemic.

#### MATERIALS AND METHODS

**Populations. (i) Blood donors.** From August to October 2001, whole-blood samples were collected from adult blood donors at the National Blood Transfusion Center at Princess Marina Hospital in Botswana's capital city, Gaborone. All blood donors were screened for the following conditions: weight loss, lung disease, tuberculosis, abdominal disease, heart disease, low or high blood pressure, kidney disease, epilepsy, diabetes mellitus, rheumatic fever, cerebrovascular accident, circulatory problems, venereal disease, allergies or asthma, goiter, jaundice, liver disease, and malaria. Potential donors exhibiting any of the conditions listed above were disqualified from donation. Additionally, a history of recent or current injections, vaccinations, medicines, or major surgery or a recent illness could exclude potential donors.

**(ii) The 2001 Sentinel Surveillance participants.** Women and men presenting at 11 representative health districts from July to September 2001 were included in this study. The women were presenting for the first time during their current pregnancies for antenatal care (ANC), and the men were presenting for symptoms suggestive of sexually transmitted infections (STIs).

This study was reviewed and approved by the Health Research Development Unit (Ethical Review Board) of the Botswana Ministry of Health.

**Sample collection and processing.** Unlysed whole-blood samples from both populations were collected in tubes containing EDTA (Becton Dickinson) to prevent clotting. Blood donor samples were collected in the afternoon and were stored at ambient temperature (10 to 20°C) until they were tested, which was completed within 36 h of blood collection. The blood samples from Sentinel Surveillance participants were collected at participating sites each morning and were transported to the Botswana-Harvard HIV Reference Laboratory at ambient temperature. These samples were labeled with a serialized code number that could not be linked to individual participants and that corresponded to numbers on abbreviated demographic questionnaires. To be included in the analysis, samples had to (i) reach the laboratory within 24 h of collection, (ii) be properly labeled, and (iii) exhibit adequate integrity (i.e., no visible clotting and/or hemolysis). All samples were processed within 12 h of arrival at the Botswana-Harvard HIV Reference Laboratory, which is within the allowable time limit stipulated by the instructions of the manufacturer.

**Sample testing and quality assurance.** Screening of all samples for HIV type 1 (HIV-1) was performed by parallel testing by enzyme-linked immunosorbent assay (ELISA) with the Murex HIV (version 1.2.0) assay (Abbott Pharmaceuticals, Inc.) and the HIV-1/HIV-2 AB-Capture ELISA system (Ortho Clinical Diagnostics, Inc.) to detect the presence of HIV-1 and HIV-2 antibodies.

A FACSCount fluorescence-activated cell sorter (FACS) system (Becton Dickinson) was used to enumerate absolute values for CD4 cells (helper and inducer T lymphocytes) and CD8 cells (suppressor and cytotoxic T lymphocytes) as well as the CD4<sup>+</sup>-to-CD8<sup>+</sup> ratios for each sample.

FACSCount instrument performance was ensured by the following: (i) testing of numerous specimens for the concordance of the results by using a separate FACSCount instrument at an independent laboratory, (ii) regular servicing and maintenance of the FACSCount machine by Becton Dickinson, and (iii) internal validation of the FACSCount machine against the Botswana-Harvard HIV Reference Laboratory's FACS Calibur flow cytometer. The performance of the Botswana-Harvard HIV Reference Laboratory is monitored regularly, as it par-

ticipates in the quality assurance program of the United Kingdom National External Quality Assessment Scheme for Leukocyte Immunophenotyping.

**Demographic data.** Each Sentinel Surveillance participant was asked to complete an anonymous questionnaire that provided demographic information (including age, gender, and the location of the participating health facility) for the purposes of this study. Demographic data (age and gender) for the blood donors were retrieved from blood donor registration records.

**Statistical analysis.** Medians, means, ranges, 2.5th to 97.5th percentiles, standard deviations, and 95% confidence intervals (CIs) for the mean were calculated for each immunohematological parameter. The nonparametric Wilcoxon rank-sum test was used to compare the distributions of the immunohematological parameters by gender and population sampled.

#### RESULTS

A total of 547 blood donor samples were collected, and of these, 437 (80%) tested HIV negative by the dual ELISAs. Of the HIV-negative blood donors, 143 were female and 294 were male.

A total of 589 samples were collected from Sentinel Surveillance participants. Of these, 499 were suitable for T-lymphocyte subset enumeration, and 251 (50%) of these 499 samples were HIV negative. A total of 207 Sentinel Surveillance participants were female and 44 were male. The samples from Sentinel Surveillance participants were representative of both urban (61%) and rural (39%) populations from 11 health districts located throughout Botswana.

The median age of the HIV-negative female donors was 27 years (interquartile range, 19 to 35 years), and that for the HIV-negative males was 29 years (interquartile range, 23 to 36 years). The median age of the HIV-negative female antenatal clinic attendees was 23 years (interquartile range, 20 to 28 years), and that for HIV-negative males attending a clinic for STIs was 26 years (interquartile range, 21 to 28 years).

Summary statistics and ranges for absolute CD4<sup>+</sup> LCs, CD8<sup>+</sup> LCs, and CD4<sup>+</sup>-to-CD8<sup>+</sup> ratios are reported in Tables 1, 2, and 3, respectively. Among the HIV-negative blood donors, the median absolute CD4<sup>+</sup> LC was 726 cells/mm<sup>3</sup>, the median absolute CD8<sup>+</sup> LC was 488 cells/mm<sup>3</sup>, and the median CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio was 1.57. Among the blood donors, differences were seen by gender for CD4<sup>+</sup> LCs ( $P < 0.001$ ) and the CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio ( $P = 0.016$ ) but not CD8<sup>+</sup> LCs ( $P = 0.36$ ); women had higher median CD4<sup>+</sup> LCs and CD4<sup>+</sup>-to-CD8<sup>+</sup> ratios.

Among the Sentinel Surveillance population, the median CD4<sup>+</sup> LC was 599 cells/mm<sup>3</sup>, the median absolute CD8<sup>+</sup> LC was 434 cells/mm<sup>3</sup>, and the CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio was 1.40. No

TABLE 2. CD8<sup>+</sup> lymphocyte counts for HIV-negative adults in Botswana

Group and sex	No. of subjects	CD8 <sup>+</sup> LC (no. of cells/ml)					
		Median	Range	2.5th–97.5th percentile	Mean	SD	95% CI for mean
Blood donor							
Female	143	494	155–1,198	228–1,062	523	203	490–557
Male	294	485	90–1,573	178–994	502	205	479–526
Both	437	488	90–1,573	190–1,014	509	205	490–528
Sentinel Surveillance							
Female	207	428	138–1,457	178–791	450	171	427–474
Male	44	491	228–1,325	232–1,299	577	294	488–666
Both	251	434	138–1,457	179–985	473	203	447–498

significant differences were detected when median CD4<sup>+</sup> LCs were compared by gender ( $P = 0.58$ ). Males had significantly higher median CD8<sup>+</sup> LCs ( $P = 0.029$ ) and lower CD4<sup>+</sup>-to-CD8<sup>+</sup> ratios ( $P = 0.009$ ).

The CD4<sup>+</sup> LCs and CD4<sup>+</sup>-to-CD8<sup>+</sup> ratios were significantly higher for the male and female participants in the blood donor population (Table 4) than those for the male and female participants in the Sentinel Surveillance population.

## DISCUSSION

The primary objective of this study was to characterize CD4<sup>+</sup> LCs among representative populations of HIV-negative adults in Botswana. The median CD4<sup>+</sup> LCs for our study populations were lower than the reference values for Tanzania, Uganda, Cameroon, the Central African Republic, and Ethiopia. The median CD4<sup>+</sup> LCs for samples collected in Botswana are also lower than those for the European control group evaluated in an Ethiopian study (20, 28, 39, 40, 46) (Table 5).

Previous studies show that population differences in CD4<sup>+</sup> LC reference ranges are influenced by a variety of factors, including genetics and environmental characteristics. Howard et al. (14) found significantly different CD<sup>+</sup> LCs among healthy Asian and non-Asian populations living in the United States. In two other studies (29, 39), Dutch control groups had significantly higher CD4<sup>+</sup> LCs than the Ethiopian population investigated.

International working groups have recently addressed the concern of interlaboratory variability in absolute CD4<sup>+</sup> LC measurements (25, 33), especially when various T-lymphocyte

immunophenotyping methods are used. The absolute CD4<sup>+</sup> LCs in the present study were measured with the FACSCount system, a single-platform technology which is regarded as a reliable and robust method for the enumeration of CD4<sup>+</sup> lymphocytes (36) and with excellent performance in terms of interlaboratory variability (3, 6, 21). Two other studies that used the same technology and that were conducted with different African populations (Cameroonians and Tanzanians) reported higher CD4<sup>+</sup> LCs (Table 5). These results indicate that absolute CD4<sup>+</sup> LCs vary among different populations and classify our HIV-1-negative Botswana populations as having comparably low absolute CD4<sup>+</sup> LCs.

The median CD4<sup>+</sup> LCs among potentially representative study populations within Botswana itself vary considerably: 726 cells/mm<sup>3</sup> for blood donors and 599 cells/mm<sup>3</sup> for Sentinel Surveillance participants. These differences may be due to a multitude of factors, including host factors like gender and pregnancy, various specimen collection times, and the level of screening for possible confounding comorbid medical conditions. Recent studies show that HIV-negative females have higher average CD4<sup>+</sup> LCs than males (22, 32, 38), a finding supported by the results of our studies with both populations, particularly blood donors. Similar studies also report that CD4<sup>+</sup> LCs decline during early pregnancy in women without HIV infection (18, 37). The findings from a more recent study characterizing the effects of pregnancy on CD4<sup>+</sup> LCs (conducted with HIV-infected pregnant women) are less conclusive (41). The effect of pregnancy may have decreased the absolute CD4<sup>+</sup> LCs in the Sentinel Surveillance population, thus decreasing the differences between males and females and accen-

TABLE 3. CD4<sup>+</sup>-to-CD8<sup>+</sup> ratios for HIV-negative adults in Botswana<sup>a</sup>

Group and sex	No. of subjects	CD4 <sup>+</sup> -to-CD8 <sup>+</sup> ratio					
		Median	Range	2.5th–97.5th percentile	Mean	SD	95% CI for mean
Blood donor							
Female	143	1.66	0.59–3.86	0.89–3.18	1.72	0.57	1.62–1.81
Male	294	1.51	0.45–5.83	0.76–2.82	1.58	0.60	1.52–1.65
Both	437	1.57	0.45–5.83	0.76–2.88	1.63	0.60	1.57–1.68
Sentinel Surveillance							
Female	207	1.44	0.19–3.44	0.79–2.75	1.50	0.53	1.43–1.57
Male	44	1.18	0.25–2.63	0.26–2.51	1.29	0.57	1.11–1.46
Both	251	1.40	0.19–3.44	0.63–2.71	1.46	0.54	1.40–1.53

<sup>a</sup> CD8<sup>+</sup> LCs >2,000 cells/mm<sup>3</sup> were set equal to 2,000 cells/mm<sup>3</sup> for calculation of the CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio.

TABLE 4. Comparison of lymphocyte subsets for study populations by gender

Population and group characteristic	Subgroup median			Wilcoxon <i>P</i> value
	Blood donor	ANC	STI	
Females				
CD4 <sup>+</sup> LC (no. of cells/mm <sup>3</sup> )	786	612		<0.001
CD8 <sup>+</sup> LC (no. of cells/mm <sup>3</sup> )	494	428		0.001
CD4 <sup>+</sup> /CD8 <sup>+</sup>	1.66	1.44		<0.001
Males				
CD4 <sup>+</sup> LC (no. of cells/mm <sup>3</sup> )	698		591	0.005
CD8 <sup>+</sup> LC (no. of cells/mm <sup>3</sup> )	485		491	0.40
CD4 <sup>+</sup> /CD8 <sup>+</sup>	1.51		1.18	0.001

tuating the differences in CD4<sup>+</sup> LCs compared to those of the blood donor population.

CD4<sup>+</sup> LCs are influenced by the presence of comorbid medical conditions, such as *Mycobacterium tuberculosis* infection (42). Data derived from the Sentinel Surveillance population primarily comprise those for women presenting for ANC in early pregnancy and men presenting with symptoms referable to underlying STIs (who have a very high risk for HIV infection). Apart from these indicated conditions and the known negative test result for HIV-1, nothing is known about the underlying health status of these participants. Given the high incidence of tuberculosis in Botswana (an estimated 620 new cases per 100,000 inhabitants per year) (31), it is possible that individuals with active tuberculosis and even an acute retroviral infection (preseroconversion) may have been included in this analysis.

Large diurnal variations in CD4<sup>+</sup> LCs have been reported (10, 23, 30), and these variations may have influenced the results obtained in the present study, as sample collection times were different for the two populations: blood was drawn from Sentinel Surveillance participants in the early morning, while blood was collected from blood donor participants in the afternoon.

Because of these potential limitations, we believe that the

blood donor population is a more representative reference population. The CD4<sup>+</sup> LCs for this group are somewhat higher than those for the Sentinel Surveillance participants and are more consistent with findings from previous studies.

The absolute CD8<sup>+</sup> LCs for our study population were comparable to the absolute CD8<sup>+</sup> LCs reported for European blood donors (39). The CD8<sup>+</sup> LCs for adults in Botswana, however, are lower than those reported for other African populations. The median CD4<sup>+</sup>-to-CD8<sup>+</sup> ratio for the population in Botswana (1.18 to 1.66) therefore lies between those found for populations with high CD4<sup>+</sup> LCs and low CD8<sup>+</sup> LCs (Europe) and those with low CD4<sup>+</sup> LCs and high CD8<sup>+</sup> LCs (Ethiopia).

**Limitations.** There were several limitations to this study. All the information on the form used to screen blood donors was filled out by the blood donors and was not independently verified. It is therefore possible that some donors did not remember recent infections or the use of medications, especially herbal and over-the-counter products. In addition, recent seroconversion would not have been detected by ELISA and would have been included in the analysis of samples from HIV-negative individuals.

Our method of T-lymphocyte subset counting (with a FACS Count system) does not include the CD4<sup>+</sup> percentage, a parameter that has been shown to be less variable in some situations. The clinical utility of CD4<sup>+</sup> lymphocytes as a percentage of total lymphocytes (especially for adult populations) is less well defined, yet guidelines recommend that they be reported in clinical trials (34).

**Implications.** Our finding of low CD4<sup>+</sup> LCs among HIV-negative adults in Botswana adds to emerging data supporting the presence of significant differences in reference CD4<sup>+</sup> LCs between different populations. The pivotal role of CD4<sup>+</sup> LCs in making decisions on the initiation and monitoring of HAART in Botswana and other developing countries underscores the importance of establishing CD4 LC reference ranges for local populations.

Validation of the low CD4<sup>+</sup> LCs observed in these populations will also be important, especially as predictors of the

TABLE 5. Comparison of reported CD4 reference values from populations and technology used

Country (reference)	Population description	No. of subjects	CD4 <sup>+</sup> lymphocyte count (no. of cells/mm <sup>3</sup> )				Technology used
			Mean	SD	Median	2.5th–97.5th percentile range <sup>a</sup>	
Botswana (present study)	ANC and STI Sentinel Surveillance participants	251	626	208	599	275–1,114	FACSCount
Botswana (present study)	Blood donors	437	759	245	726	366–1,318	FACSCount
Tanzania (16)	Local population near hospital	147	980 <sup>b</sup>	310	968	372–1,588	FACSCount
Cameroon (35)	Healthy visitors or guardians at hospital	203	980 <sup>c</sup>			350–1,610	FACSCount
Uganda (19)	Visitors to AIDS information center	183	1,256 <sup>c</sup>			559–2,333	FACScan
Ethiopia (17)	Factory workers	142	775 <sup>c</sup>	225	761	366–1,235	FACScan
The Netherlands (17)		1,356	993 <sup>b</sup>	319	950	509–1,761	FACScan
Central African Republic (23)							
Male		68	927	349	851	380–1,617	FACS Calibur
Female		82	940	291	912	386–1,454	FACS Calibur

<sup>a</sup> The 95th percentile ranges are presented for all but the Tanzanian and Cameroonian populations. For the Tanzanian study we estimated the 2.5th to 97.5th percentile using the mean  $\pm$  1.96  $\times$  standard deviation. The 2.5th to 97.5th percentile range was not available for the Cameroonian study, so the range is presented.

<sup>b</sup> Comparison by gender was not reported.

<sup>c</sup> Women were reported to have higher values than men.

extent and rate of HIV disease progression. Internationally established CD4<sup>+</sup> LC cutoff values for the management of HIV disease are derived from comparably higher baseline CD4<sup>+</sup> LCs, yet the functional significance of smaller pools of CD4<sup>+</sup> cells at the outset of HIV disease remains unclear. A cohort of individuals in the early stages of HIV infection (who are not yet receiving antiretroviral treatment) should be monitored longitudinally, an approach that is in the early stages of implementation in Botswana.

The increasing reliance on CD4<sup>+</sup> LCs as a means to govern the initiation and monitoring of HAART among HIV-infected individuals in resource-limited settings makes it imperative that standardized, precise, and affordable methodologies for CD4<sup>+</sup> LC determination be used (16, 17). In addition, implementation of uniform quality control procedures for routine clinical assays will be important (3, 24) to ensure the inter- and intralaboratory comparabilities of baseline and longitudinal CD4<sup>+</sup> LC measurements.

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